A THREE PHASE STUDY INVOLVING THE EXPERIMENTAL SYNTHESIS AND CHARACTERISATION OF NANO-PARTICULATE SEALERS, THEIR CYTOTOXIC EVALUATION FOLLOWED BY ANALYSIS OF THEIR ANTIMICROBIAL EFFICACY IN AN EX-VIVO MODEL UNDER CONFOCAL LASER SCANNING MICROSCOPE.

Dissertation submitted to

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In partial fulfilment for the Degree of

MASTER OF DENTAL SURGERY

BRANCH IV

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CERTIFICATE

This is to certify that this dissertation titled **"A three phase study involving the experimental synthesis and characterization of nano-particulate sealers, their cytotoxic evaluation followed by analysis of their antimicrobial efficacy in an ex-vivo model under confocal laser scanning microscope"** is a bonafide record of work done by **Dr. GAYATHRI VELUSAMY** under my guidance and to my satisfaction during her postgraduate study period, 2013 – 2016. This dissertation is submitted to **THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY**, in partial fulfilment for the award of the degree of Master of Dental Surgery in Conservative Dentistry and Endodontics, Branch IV. It has not been submitted (partially or fully) for the award of any other degree or diploma.

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CONTENTS

INTRODUCTION

Endodontic treatment is directed towards the prevention and control of pulp and periradicular infections, which is achieved through instrumentation, irrigation and intracanal medication. Nevertheless, these procedures do not completely eliminate the microorganisms from the root canal; hence obturation of the root canal space with a suitable material that possesses antibacterial property would be beneficial in further reducing the number of residual microbes[1].Root canal sealers fill the irregularities and minor discrepencies between core filling material and canal wall thus ensuring a three dimensional seal. Antimicrobial properties of these sealers together with hermetic seal obtained with obturation material ensure elimination of microbes[2] as well as prevent reinfection.

The materials used for endodontic procedures must be chosen with care so that potential periapical reactions can be avoided or reduced. The egress of chemical components from the obturation material through the apical foramen might lead to certain unfavorable periapical tissue reactions[3-5]. This can be very significant especially for root canal sealers in the event of the sealer extruding periapically. Ideally, root canal sealers should be biocompatible and have satisfactoryphysico-chemical and antimicrobial properties. Achieving a biocompatible filling material is a challenging task for the success of endodontic treatment. **Hence along with antimicrobial properties, biocompatibility of the cement sealers are highly relevant and useful in root canal treatment and worthy of evaluation[6].**

Zinc oxide-eugenol sealers have a history of successful use over an extended period of time. Since its introduction, gradual developments have been made on the chemistry and properties of ZOE sealer to improve its quality. ZOE sealers are easy to handle, exhibit lowest shrinkage of 0.14% and less dimensional change compared to

other resin based sealers[7], has long lasting antimicrobial property on a variety of microorganisms including E.faecalis suspensions and anaerobic bacteria even 7 days after mixing[8]. Their chief drawback is their solubility which will lead to microleakage thereby affecting the apical seal.

Recent advances in biomaterial sciences have introduced Nano technology into modern dentistry. Materials reduced to the nanoscale can show very different properties compared to what they exhibit on a macroscale, enabling unique applications. For example, opaque substances become transparent (e.g. copper); inert materials become catalysts (e.g. platinum); stable materials turn combustible (e.g. aluminum); solids turn into liquids at room temperature (e.g. gold); insulators become conductors (e.g. silicon). Much of the fascination with nanotechnology stems from these unique quantum and surface phenomena that exhibits at the nanoscale.

Antibacterial nanoparticulates are found to have higher antibacterial activity than antibacterial powders because of the higher surface area and charge density of nanoparticulates, which enable them to achieve a greater degree of interaction with the negatively charged surface of bacterial cells [9].ZnO nanostructures show better antibacterial properties due to their surface enhancement and it can be further enhanced by doping some elements like Ag, Au, Ni and Co in ZnO matrix[10].

The endodontic microflora is typically polymicrobial consisting of gram positive and gram negative bacteria. Enterococcus faecalis remains to be the most frequently identified species in canals of root filled teeth with periapical lesions[11-13]. The ability of E.faecalis to cause periapical disease and chronic failure of an endodontically treated teeth, to survive prolonged periods of nutritional deprivation / harsh environment and to resist the most common inter-appointment medicaments[14-

[16]are due to its various virulence factors. Hence, elimination of E.faecalis in experimental studies is considered to be an indicator of the efficacy of the test material.

Conventional procedures such as histologic section, scanning electron microscopy, transmission electron microscopy and microbiological sampling methods can be used for assessing the presence of bacteria in the root canal. Each one of these methods has its advantages and disadvantages. Recently Weiger et al introduced fluorescent staining to show viability of bacteria on infected dentin[17]. Further, combining fluorescent viability staining with confocal laser scanning microscopy (CLSM) made it possible to visualize the distribution of live and dead bacteria directly within the tubules which closely mimics the clinical situation[18].

With the advent of such improved technology, the efficacy of various root canal materials can be studied and quantified with precision. Additionally, visualization of the live and dead bacteria within the tubules can enable us to understand the intricacies involved in bacterial penetration and the antimicrobial effect required to counteract it based on its potency.

Thus the purpose of this study was

1. To synthesize experimental nanosealers ***zinc oxide nanopowders*** and ***zinc oxide: silver nanopowders*** by hydro-thermal method and characterize their surface morphology by UV visible spectroscopy and Transmission Electron Microscopic ananlysis.
2. To assess the cytotoxicity of synthesized experimental nanosealers on human osteoblastic-like MG63 cell lines using MTT Assay.

3. To evaluate the antimicrobial efficacy of synthesized experimental naosealers on the Enterococcus faecalis biofilm model grown on root canals of extracted single rooted teeth using confocal laser scanning microscopy.

AIMS & OBJECTIVES

The purpose of the current study was to evaluate the cytotoxicity and antimicrobial efficacy of experimentally synthesized Zinc oxide nanopowders and Zinc oxide:Silver nanopowders compared with Zinc oxide Eugenol sealer against Enterococcus faecalis, by assessing the bacterial viability using Confocal laser scanning microscopy.

REVIEW OF LITERATURE

ANTIMICROBIAL EFFICACY OF ZNO SEALERS

Saha et al (2010)[19] assessed the antimicrobial activity of three root canal sealers Endomethasone, AH 26 and Apexit against seven strains of bacteria (aerobes, facultative and obligate anaerobes) known to be common isolates in necrotic pulps and endodontic lesions at various time intervals using the agar diffusion test and concluded that Zinc oxide eugenol based root canal sealer produced largest inhibitory zones followed by epoxy resin based sealer and least by calcium hydroxide based root canal sealer.

Anjali Kaiwar et al (2012)[20] assessed the antimicrobial activity of Endoflas FS, Metapex, AH 26 and AH Plus sealers on *Enterococcus faecalis* using Agar diffusion test and showed that Endoflas FS showed significantly greater antimicrobial effect against *E.faecalis* followed by Metapex, whereas resin-based sealer showed no antimicrobial activity on *Enterococcus faecalis*.

Kothari A et al (2013)[21] evaluated the invitro antimicrobial activity of five root canal sealers namely Zinc Oxide Eugenol, Endoflas FS, Endomethasone, AH plus and Sealapex against *Enterococcus faecalis* using Agar diffusion method. The authors concluded that Endomethasone showed significantly greater antimicrobial effect against *E.faecalis* followed by Zinc Oxide Eugenol and Endoflas FS, whereas Sealapex was less effective and AH plus showed no antimicrobial activity.

Arora et al (2014)[22] evaluated the antimicrobial properties of three endodontic sealers Sealapex, Endoflas FS and AH plus against *Enterococcus faecalis* using Agar diffusion test and concluded that the antibacterial activity of Endofals FS was highest followed by AH plus and Sealapex.

Nirupama et al (2014)[23] evaluated the antimicrobial activity of four endodontic sealers, AH Plus, Tubliseal EWT, EndoRez and iRoot SP against three different microorganisms *E.faecalis*, *C.albicans* and *S.aureus* by direct contact test. The results showed that AH Plus and iRoot SP had significantly higher antimicrobial activity against *E.faecalis*. AH Plus and TUbliseal EWT showed significantly higher antimicrobial activity against *C.albicans* and *S.aureus* compared to iRoot SP and EndoRez. EndoRez showed the least antimicrobial activity against all the three microorganisms. In conclusion AH Plus had significantly higher antimicrobial activity against *E.faecalis, C.albicans* and *S.aureus*.

NANO ZINC OXIDE SEALER

Shrestha et al (2010)[24] evaluated the efficacy of chitosan nanoparticle and zinc oxide nanoparticle in disinfecting and disrupting biofilm bacteria and the long term efficacy of these nanoparticulates following aging against *enterococcus faecalis* in planktonic and biofilm forms using microbiologic methods and confocal laser scanning microscopy analysis. The confocal microscopy images showed predominantly dead bacteria and significant reduction in the thickness of biofilm after nanoparticulate treatment and both the groups were found to retain their antibacterial properties after aging for 90 days. The authors concluded that CS-np and ZnO-np possess a potential antibiofilm capability.

Guerreiro-Tanomaru et al (2013)[25] evaluated the pH and antimicrobial activity of micro or nanoparticulate zinc oxide (ZnO) pastes with or without calcium hydroxide (CH) against *Enterococcus faecalis*, *Candida albicans*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Kocuria rhizophila* using Agar diffusion test. The medications evaluated were: Microparticulate ZnO + polyethylene glycol (PEG) 400;

Nanoparticulate ZnO + PEG 400; PEG 400; CH+Microparticulate ZnO + PEG 400 and CH + Nanoparticulate ZnO + PEG 400. The highest pH values were found for CH+ZnO, with higher values for nanoparticulate ZnO after 12 hours and 21 days ($p<0.05$). CH+ZnO medication promoted higher growth inhibition against P. aeruginosa and lower against *E.faecalis.* Calcium hydroxide pastes have higher pH and antimicrobial activity when associated with either micro- or nanoparticulate zinc oxide.

Javidi et al (2014)[26] evaluated the sealing ability of three types of new experimental nano-ZOE-based sealer with two other sealers AH 26 and micro-sized zinc oxide eugenol sealer. Microleakage in AH26 groups was significantly more than that in three groups of ZnO nano‑particles. Apical microleakage of ZnO micro‑powders was significantly more than that of all the materials, but the sealing ability of ZnO nano‑powder sealers did not differ significantly. In conclusion this study showed that the synthesized ZnO nano‑powder sealers are suitable for use as a nano‑sealer in root canal therapy to prevent leakage; however, further studies should be carried out to verify their safety.

NANO SILVER

Lotfi et al (2011)[27] in their study compared the antibacterial efficacy of nanosilver (NS), chlorhexidine gluconate (CHX) and sodium hypochlorite (NaOCl) against *Enterococcus faecalis* using agar diffusion methods and showed that NS in a remarkably lower concentration would possess the same bactericidal effect as 5.25% NaOCl. However, the zones of inhibition for 2% CHX were significantly larger than those seen around the filter papers saturated with undiluted NaOCl and NS ($p < 0.001$ for both).

Makkar et al (2014)[28] evaluated the antibacterial properties of silver nano particle based irrigant as endodontic root canal irrigant against *Enterococcus faecalis* using agar diffusion method and the results showed the number of colony forming units dropped to zero after 3 minutes contact time with silver nano particle based irrigant and showed large inhibition zones as compared to NaOCl 3% group, hence concluded Silver Nano Particle based irrigant as an effective endodontic irrigant.

SYNTHESIS OF NANOPARTICLES

Pavel et al (2012)[29] in their study synthesized Ag-ZnO nanoparticles by two different microwave techniques from silver nitrate and zinc nitrate and characterized by X-ray diffraction and Scanning electron microscopy method. The antibacterial activity was evaluated by inhibition zone test against *E.coli*, *Staphylococcus aureus* and *Candida albicans* which showed observable antibacterial activity against *S.aureus* and *C.albicans*.

Vijayakumar et al (2013)[30] in their study synthesized silver-doped zinc oxide (Ag:ZnO) nanocomposite material using chemical co-precipitation method in which 0.2M zinc chloride and 0.001 M silver nitrate co-precipitated with 25% ammonia solution by pulse mode dispersion using ultrasonicator. The resulted Ag:ZnO nanocomposite was structurally and optically characterized using X-ray diffraction , UV-Visible spectrophotometer, Fourier transform infrared spectroscopy (FT-IR), Scanning electron microscopy and Transmission electron microscopy analysis.

Chauhan et al (2015)[31] investigated the biological synthesis, characterization, antimicrobial and synergistic effect of silver and zinc oxide nanoparticles against clinical pathogens using Pichia fermentans JA2 isolated from spoiled fruit pulp. The

biological synthesis of silver and zinc oxide nanoparticles is a novel and cost-effective approach over harmful chemical synthesis techniques. Antimicrobial efficacy of these nanoparticles was tested against medically important Gram positive, Gram negative and fungal pathogenic microorganisms (*Escherichia coli, Klebsiella pneumonia, Salmonella sp, Staphylococcus aureus, Enterococci sp, Pseudomonas aeruginosa, Shigella sp, Proteus mirabilis, Candida tropicalis, Fusarium sp, Aspergillus terreus strain*) and found that the silver nanoparticles inhibited most of the Gram negative clinical pathogens whereas zinc oxide nanoparticles were able to inhibit only Pseudomonas aeruginosa.

ANTIMICROBIAL ACTIVITY OF NANO ZnO and Ag/ZnO

Hernandez-Sierra et al (2008)[32]compared the bactericidal and bacteriostatic effects of nanoparticles of silver, zinc oxide and gold on *Streptococcus mutans*. Using liquid dilution method the minimum inhibitory concentration and minimum bactericidal concentrations for silver, zinc oxide and gold was found. The study showed a higher antimicrobial effect against *S.mutans* of Silver nanoparticles at lower concentrations than gold or Zinc which would allow achieving important clinical effects with a reduced toxicity.

Kishen et al (2008)[33] investigated the antibacterial and antibiofilm efficacy of cationic nanoparticulates (zinc oxide nanoparticle ZnO-NP and chitosan nanoparticle CS-NP) mixed with zinc oxide eugenol based sealer for root canal disinfection. ZnO-NP, CS-NP, ZnO/CS-NP and CS-layer-ZnO-NP were tested. The study showed that the incorporation of nanoparticulates did not alter the flow characteristics of sealer but improved the direct antibacterial property and the ability to leach out antibacterial components and there was a significant reduction in the adherence of *Enterococcus*

faecalis to nanoparticulates-treated dentin highlighting the potential advantage of ZnO-NP and CS-NP to inhibit bacterial recolonization in root canals and to improve the antibacterial capabilities of endodontic sealers.

Jafari et al (2011)[34] evaluated the antibacterial activity of nano zinc oxide combined with silver nanocrystals synthesized via wet method (oxalate decomposition method) against *Escherichia coli, Salmonella galinarium, Staphylococcus aureus, Pseudomonas auerоginosa* and *Bacillus subtilis* using disc diffusion test and agar dilution test also with determination of minimum inhibitory concentration and minimum bactericidal concentration. The authors concluded that Ag/ZnO nanocrystals have great antimicrobial effect against all of the strains and just combination of zinc oxide and silver nanocrystals increased their bactericidal effect.

Jide Zhang (2011)[35] in his study investigated a precipitation method to prepare nanoscale zinc oxide (ZnO) as the precursor of silver coated antibacterial nanocomposite and subsequently silver loaded zinc oxide nanocomposites (ZnO/Ag) were prepared by hydrolysis method and showed that silver loaded ZnO prepared by hydrolysis method exhibited strong antibacterial activity on *Escherichia coli* and *Staphylococcus aureus* using inhibition zone test.

Karunakaran et al (2011)[36] evaluated the optical, electrical, photocatalytic and bactericidal properties of microwave synthesized nanocrystalline Ag:ZnO and ZnO by X-ray diffraction , Energy dispersive X-ray spectroscopy and scanning electron microscopy methods. The authors concluded that the microwave synthesized ZnO exhibits larger bactericidal activity than the commercial ZnO nanoparticles and doping with Ag by microwave method improves the antibacterial and photocatalytic activities.

Rad et al (2013)[37] investigated the microleakage and antibacterial properties of the experimental ZnO nanopowders and ZnO:Ag nanopowders synthesized via sol gel method and compared with zinc oxide eugenol sealer and epoxy resin sealer (AH 26). The authors concluded that the prepared ZnO and ZnO:Ag nanopowders exhibit a better microleakage and antibacterial properties than the AH 26 and ZOE sealers and therefore are more suitable filling materials to be used as sealer in root canal treatment.

CYTOTOXICITY

Ho et al (2006)[38]evaluated the mechanisms of cytotoxicity of eugenol in human osteoblastic cell line U2OS in vitro using cell proliferation assay by adding antioxidants catalase, superoxide dismutases and N-acetyl L-cysteine. The results showed that eugenol demonstrated a cytotoxic effect to U2OS cells in a dose-dependent manner and addition of NAC extracellularly protected the cells from eugenol-induced cytotoxicity, neither SOD nor catalase provided any protective effects on eugenol-induced cytotoxicity. Thus they concluded that eugenol has significant potential for periapical toxicity and the inhibitory effects of eugenol on U2OS cells were associated with glutathione levels.

Huang et al (2010)[39] investigated the effects of an epoxy resin-based sealer AH26, zinc oxide-eugenol based sealer Canals and a paste sealer N2 on the expression of ALP in human osteoblastic cell line U2OS cells. Cytotoxicity was measured using Almar blue dye assay and Gene expression of ALP was examined by using reverse transcription-polymerase chain reaction and ALP activity by susbstrate assay. The results showed that AH26, Canals and N2 were cytotoxic to U2OS cells in a concentration dependent manner, the exposure of U2OS cells to AH26 and N2 resulted in the down regulation of ALP mRNA gene expression and ALP activity was significantly suppressed by 3 root

canal sealers. They concluded that the inhibition of ALP expression might play an important role in the pathogenesis of root canal sealer induced periapical bone destruction and care should be taken to avoid inadvertent extrusion of the root canal sealers in clinical treatment.

Noriko MUTOH and Nobuyuki TANI-ISHII (2011)[40] established an experimental animal mode (the molars of wistar rats were extracted and repositioned in the original socket after application of the sealers on the root apices) to evaluate the biocompatibility of new paste type Zinc oxide-eugenol sealer with those of conventional powder/liquid ZOE and eugenol free sealers. Mild inflammation occurred in the periapical tissue of the replanted teeth with both ZOE sealers on day 7, whereas the eugenol-free sealer induced severe inflammation. On day 14, the lesions induced by all types of sealers were healed and replaced predominantly by fibrous connective tissue. Thus, all sealers showed high biocompatibility although the extent of inflammatory reaction during the early stages varied depending on the types of materials

Hegde et al (2011)[41] evaluated the cytotoxicity of three sealers, Pulpdent Root canal sealer (eugenol based), Endomethasone and AH 26 on human gingival fibroblasts using MTT assay at 24 hours, 7th day and 14th day and concluded that Pulpdent root canal sealer was less cytotoxic than Endomethasone and AH 26.

Scelza et al (2011)[42] performed a study to compare the cytotoxicity of four endodontic sealers (Sealapex, Pulp canal sealer EWT, Real seal and MTA Fillapex) on human primary osteoblasts using MTT assay at 1st and 7th day after mixing. They concluded that all endodontic sealers had strong cytotoxicity 24 h after mixing and at a longer

setting period(7 days) Pulp Canal Sealer achieved levels of cytocompatibility similar to the control group.

Rezende et al (2013)[43]evaluated the cytotoxicity and genotoxicity of zinc oxide and eugenol at different proportions using Allium cepa assay at the concentration of 1 drop of eugenol and 1 portion of zinc oxide cement (Treatment I) and twice the concentration of eugenol (Treatment II). It was concluded that the change in size, thickness and color of the treated roots were caused by the cytotoxic activity of the zinc oxide-eugenol cement in the concentration of one or two drops of eugenol and the chromosomal aberrations such as anaphase bridges suggested the genotoxic effect of this substance in the concentration of two drops of eugenol for each portion of zinc oxide.

Silva et al (2013)[44]investigated the effects of 8 root canal sealers (AH plus, Epiphany, Endomethasone N, EndoREZ, MTA fillapex, Pulp Canal Sealer EWT, Roeko Seal and Sealapex) on the cytotoxicity of 3T3 fibroblasts during a period of 5 weeks using MTT assay. They showed that RoekoSeal had no cytotoxic effect both freshly mixed and in the other tested time points, MTA Fillapex remained moderately cytotoxic after the end of experimental period and all other tested sealers exhibited varying degrees of cytotoxicity mainly in fresh conditions.

Pawinska et al (2015)[45] performed a study to comparatively assess the toxic action of root canal sealers Epiphany, Endomethasone N, Tubliseal, Sealapex and GuttaFlow on human gingival fibroblasts after setting using MTT assay and concluded that only Epiphany and Sealapex were highly toxic.

E.FAECALIS

Love R.M (2001)[46] proposed a study to identify a possible mechanism that would explain how *E.faecalis* could survive and grow within dentinal tubules and reinfect an obturated root canal using micro titre well experiments. It was postulated that in failed endodontically treated teeth, *E.faecalis* cells remained viable and maintain the capability to invade dentinal tubules and adhere to collagen in the presence of human serum.

Sedgley et al (2005)[47] studied whether *Enterococcus faecalis* can survive long-term entombment in root filled teeth without additional nutrients using culture, PCR and histological methods. The results showed that *Enterococcus faecalis* inoculated into root canals maintained viability for 12-months ex vivo. The clinical implications are that viable *E. faecalis* entombed at the time of root filling could provide a long-term nidus for subsequent infection if the opportunity arises.

Chivatxaranukul et al (2008)[48] investigated the dentinal tubule invasion and the predilection of *Enterococcus faecalis* for dentinal tubule walls using scanning electron microscopy. It was found that *E.faecalis* readily invaded dentinal tubules even though the organism seemed to have low affinity for tubule walls and the adherence was higher in the less mineralized inner dentine than outer dentine. Therefore, the initial colonization of dentinal tubules by *E.faecalis* may be primarily dependent on other factors such as the environmental conditions.

Guerreiro-Tanomaru et al (2013)[49] evaluated the influence of different substrates-bovine dentin, gutta-percha, hydroxyapatite and bovine bone on the development of *E.faecalis* biofilm during 2 evaluation periods (14 or 21 days) using confocal laser

scanning microscopy. It was concluded that *E. faecalis* was able to develop biofilm on all the substrates tested during induction periods of 14 days and 21 days; hydroxyapatite was the substrate with the best conditions for biofilm development.

CONFOCAL LASER SCANNING ELECTRON MICROSCOPE

Zapata et al (2008)[50] explored the potential of Confocal Laser Scanning Microscopy for in situ identification of live and dead bacteria inside infected dentinal tubules and to describe the distribution and vitality of the bacteria *Enterococcus faecalis* in infected dentin of bovine origin by the use of immunofluorescence technique. They found that the viability and distribution of live and dead of bacteria in infected dentin can be effectively determined in situ by CLSM using Fluorescein diacetate/ Propidium Iodide and acridine orange for staining and have the potential for endodontic and cariology research

Parmar et al (2011)[51] assessed whether the application of live/dead staining to the human ex vivo dentinal tubule, infected with *E.faecalis* and treated with calcium hydroxide dressing, has the capacity to differentially image and quantify viable and non-viable bacteria within mineralized tissue by confocal laser scanning microscopy. They showed that the technique employed, provided a convenient and reproducible approach for assessing viability of the bacteria and the extent of bacterial penetration into the dentinal tubules.

MATERIALS & METHODS

ARMAMENTARIUM

- Diamond saw
- Endomotor (X-Smart, Dentsply Maillefer, Japan)
- Protaper files (Dentsply Maillefer, Switzerland)
- K files (Mani, Japan)
- Laminar air flow chamber
- Incubator (NSW, India)
- Test tubes (Borosil 27ml)
- Beaker (Borosil 50 ml/100 ml)
- Micropipette (Eppendorf)
- Microcentrifuge tube 1.5ml
- 5ml disposable syringe (Dispovan)
- Spirit lamp
- Glass slab
- Mixing spatula
- Tweezer
- Condenser/burnisher
- Lentulo spirals (Dentsply Maillefer, India)
- Spreaders (Mani, India)
- Autoclave (Unique clave C-79, Confident)
- Humidity chamber
- Hard tissue microtome (Leica, Germany)
- Confocal laser scanning microscope (Zeiss, LSM 510 META, Germany)

MATERIALS USED

- Nano ZnO powder (PSG Institute of Advanced Studies, Coimbatore, India)
- Nano ZnO/Ag powder (PSG IAS, Coimbatore, India)
- Zinc oxide Eugenol sealer (powder/liquid)
- Gutta-percha (Diadent, India)
- Absorbent paper points (Diadent, India)
- Saline (0.9% w/v sodium chloride injection, NS, Baxter, India)
- Sodium hypochlorite (Prime dental, India)
- Ethylene Diamine Tetra Acetic Acid (Dentsply Maillefer, USA)
- Agar
- Brain heart infusion broth (Himedia, India)
- Ethanol
- Enterococcus faecalis (MTCC 439 equivalent to ATCC 35550, Microbial type culture collection and Gene bank, Chandigarh)
- Fluorescent dyes – Fluorescein diacetate and Propidium iodide (Sigma, USA)
- Distilled water
- Self-cure clear acrylic
- Rubber base impression material
- Human osteoblast cell lines – MG63 cells

PHASE I – TEETH SAMPLE PREPARATION AND INFECTION OF ROOT CANALS

TEETH SELECTION AND PREPARATION:

Twenty one single-rooted human mandibular premolars with closed apices, extracted for orthodontic reasons were used in this study. The teeth were cleaned of superficial debris & calculus using ultrasonic scaler and tissue tag were removed by storing teeth in 3% sodium hypochlorite for 2 hrs. After cleaning, the teeth were stored in normal saline to prevent dehydration before use. Each tooth was radiographed to confirm the presence of a single patent canal. The tooth specimens were sectioned below the cemento-enamel junction with a slow speed diamond disc under water cooling to obtain a standardized tooth length 12 mm**(Figure 1).** The canals were accessed, and initially a size #10 Stainless Steel (SS) K was file inserted into the canal until the file tip was just visible at the apical foramen. The working length (WL) was kept 1mm short of the apical foramen.

The roots were prepared using Protaper files (Dentsply Maillefer,Switzerland) upto size F3. The canals were irrigated with sodium hypochlorite between each instrument and 17% EDTA (Dentsply Maillefer, USA) as a final irrigant to remove the smear layer. All the roots were then washed and stored in saline.

STERILIZATION OF TEETH

All the prepared teeth were packed in suitable autoclave pouches and autoclaved at 121^{o}C.

CUSTOMIZED MODEL FOR BACTERIAL INOCULATION OF TEETH

A customized model was assembled for each tooth for the subsequent bacterial inoculation and incubation. Molten Agar was expressed into three fourth of 1.5 mL micro-centrifugal tubes and allowed to cool. Once the agar reached the gel stage the teeth were embedded in the agar up to the junction of middle and coronal third of the root**(Figure 2)**. After mounting each tooth in centrifugal tube, all tubes were placed in the stand ready for bacterial inoculation**(Figure 3).**

REVIVAL OF LYOPHILISED CULTURE

The ampule containing Enterococcus faecalis (MTCC 439, Microbial type culture collection and Gene bank, Chandigarh)**(Figure 4)** was wiped with alcohol and was opened in a biological safety cabinet designed to protect against inhalation of aerosols and to protect the ampule from external contamination. The culture was transferred aseptically and a few drops of the Brain Heart Infusion broth were added to the dried culture using a Pasteur pipette. Brain Heart Infusion broth was prepared by adding 3.75g of powder having the composition of infusion from beef heart and calf brain, disodium hydrogen phosphate, glucose, peptone, and sodium chloride in 100 ml of distilled water and autoclaved at 121°c for 15 minutes. The contents were mixed well, avoiding frothing, and the suspension was transferred to test tubes and plates of containing the media. The organisms were incubated (Incubator - NSW, India) at 37º C and under the appropriate gaseous conditions **(Figure 5).**

BACTERIAL GROWTH

A 24 hour pure culture suspension of E.faecalis (MTCC 439, Microbial type culture collection and Gene bank, Chandigarh) was cultivated in brain-heart infusion (BHI) broth.

BACTERIAL INFECTION OF ROOT CANALS:

Each root was inoculated with E.faecalis by placing the suspension in the root canal of each tooth with a micropipette. Approximately 0.5µL of the suspension was placed in every tooth sample**(Figure 6).** All specimens were incubated aerobically at 37°C for 21 days. Fresh BHI broth was supplemented into the root canals weekly to ensure viability of bacteria**(Figure 7).**

Figure 1: De-coronated teeth samples

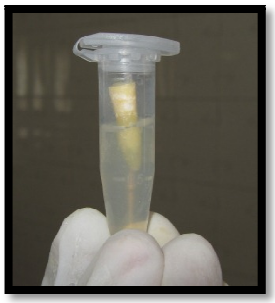


Figure 2: Customized agar mounted teeth model for root samples

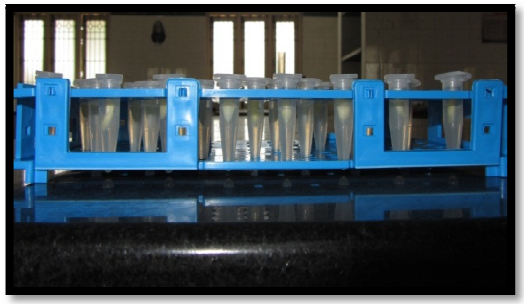


Figure 3: Centrifugal tubes containing placed in stand

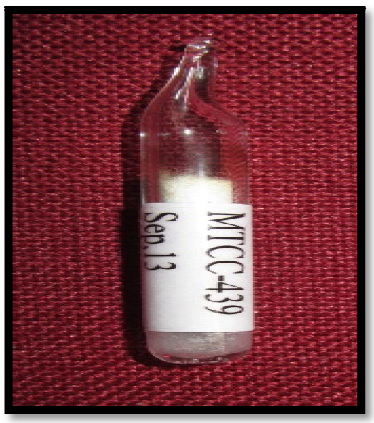


Figure 4: Enterococcus Faecalis (MTCC 439)

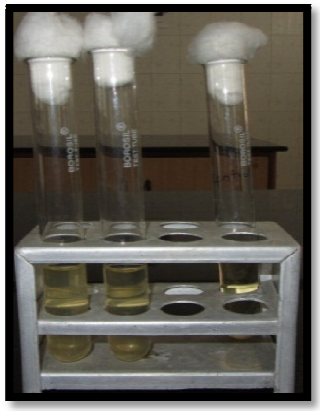


Figure 5: Prepared E.faecalis broth

Figure 6: Inoculation of root samples with 0.5µL of bacterial suspension

Figure 7: Incubator

PHASE II – SYNTHESIS OF EXPERIMENTAL NANO SEALER

Zinc chloride (98%, Merck), Potassium hydroxide (98%, Merck), Sodium borohydride (98%, Merck), Tri-sodium citrate (98%, Merck), Silver nitrate (99.9%, Merck) and 3-aminopropyl-trimethoxysilane (APTMS) (99%, Aldrich) were obtained and all the chemicals were used as such without any further purification. Ultrapure water with a resistivity of 18.2 MΩ.cm was used (Millipore Milli-Q system) for the synthesis.

SYNTHESIS OF ZnO NANORODS:

The ZnO nanorods used in this study were prepared according to hydrothermal method. In brief, 10 mL of 2 mM KOH solution was added dropwise into the 20 mL of 1 mM $ZnCl_2$ solution and stirred vigorously. 5mL of 0.5 mM CTAB (Cetyl Tetra Ammonium Bromide) solution was added to the stirring solution and white floccules appeared immediately reflecting the formation of zinc hydroxide. After 3 hours of continued stirring, the solution was transferred into a Teflon-lined stainless steel autoclave and filled by distilled water up to 80% volume. Hydrothermal treatments were carried out at 120˚C for 5 h. After the process, autoclave was allowed to cool down naturally and white precipitates of ZnO nanorods were separated via centrifugation, and washed thoroughly using distilled water and ethanol to remove impurities. Finally, the ZnO nanorods were dried at 80°C in hot air oven for 8 hours.

SYNTHESIS OF HYBRID ZnO@AG CORE-SHELL NANORODS:

For the functionalization of ZnO nanorods, 2 mg of the as synthesised ZnO nanorods were weighed and dispersed with 10 mL ultrapure water. 50 µL of 1M 3-aminopropyl-trimethoxysilane (APTMS) was added to the dispersion and stirred for 12 hours continuously in round bottom flask using magnetic stirrer. After completion of functionalization reaction, the precipitate was separated using centrifugation process at the rate of 5000 rpm for 10 minutes and washed using distilled water and ethanol several times, and then dried at 60°C. 10 mL of 7.5 M triethyl amine (TEA) solution was taken in a round bottom flask and 0.2 g of APTMS functionalised ZnO nanorods were added and stirred for 30 minutes using magnetic stirrer. 2 mL of 0.6 M of $AgNO_3$ solution was added dropwise to the mixture. A dark brown precipitate was formed

immediately after the addition indicating the formation of ZnO@Ag core-shell nanorods. 100 mL of distilled water was added to the dark brown precipitate and solution was left undisturbed for 5 hours for seed growth. The precipitate was then separated and purified carefully using distilled water and ethanol several times using centrifuge and vacuum filtration to remove impurities. Further, the materials were air dried at 80°C for overnight in hot air oven. The purified hybrid ZnO@Ag core-shell nanorods were further used for dental applications.

$$Zn^{2+}_{(aq)} + 4OH^-_{(aq)} \longrightarrow Zn(OH)_4^{2-}_{(aq)} \quad \text{..... (1)}$$

$$Zn(OH)_4^{2-}_{(aq)} + CTAB \xrightarrow{\text{Hydrothermal treatment (120°C, 5hrs)}} \text{ZnO nanorods (White)} \quad \text{..... (2)}$$

$$\text{ZnO nanorods} + TEA_{(aq)} \xrightarrow{Ag^+} \text{ZnO@Ag core-shell nanorods (Black)}$$

$$(Zn\text{---}O \cdots Ag) \quad \text{..... (3)}$$

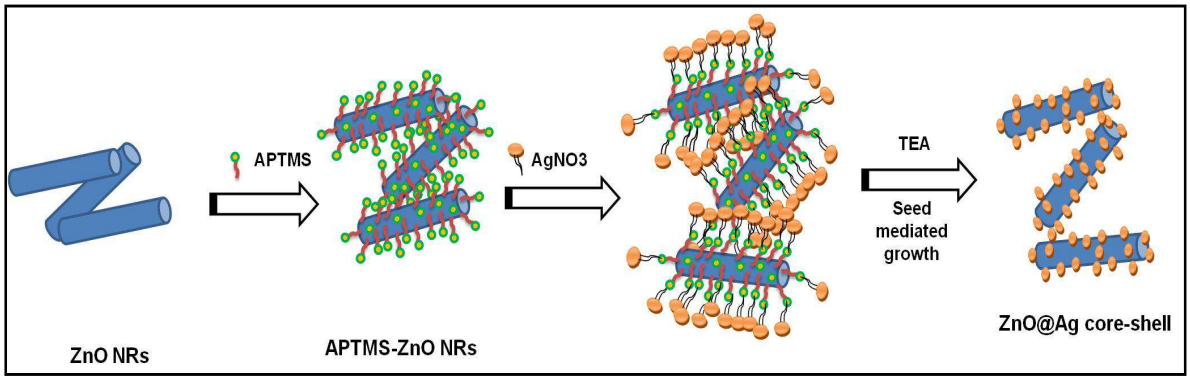


Figure 8: Schematic representation of the synthesis of ZnO@Ag core-shell nanoparticles

PHASE II - CHARACTERIZATION OF SYNTHESIZED NANO SEALERS

UV VISIBLE SPECTROSCOPY

The synthesised ZnO and ZnO@Ag nano materials were characterized using UV visible spectroscopy and Transmission Electron Microscopy. The ZnO nanorods showed the characteristic peak at 370 nm corresponding to the ground excitonic peak of pure ZnO nanorods thus confirming the purity of the synthesized ZnO nanorods. ZnO@Ag core-shell hybrid nanostructures showed existence of excitonic peak (360 nm) and the characteristic surface plasmon peak corresponding to Ag nanoclusters at 270 nm and 420 nm, thus confirming the formation of hybrid core-shell structures**(Figure 11).**

TRANSMISSION ELECTRON MICROSCOPY

High Resolution Transmission Electron Microscopic analysis was performed in order to investigate the morphological and structural properties of hybrid ZnO@Ag core-shell nanorods. **Figure.12** reveals the typical TEM images of hybrid ZnO@Ag core-shell nanorods. High resolution image of hybrid core-shell nanorods (Fig. 12b) illustrates the uniform distribution of Ag nanoclusters on the surface of ZnO nanorods confirming the formation of core-shell nanorods with hetero-junctions. Lattice mismatch between ZnO nanorods and Ag resulted in the growth of Ag nanoclusters on the surface of ZnO nanorods. Interestingly, Ag nanoclusters decorated on ZnO nanorod surface was found to be grown without any aggregation with an average particle size of ~7 ± 0.5 nm, proving the effectiveness of the synthetic process.

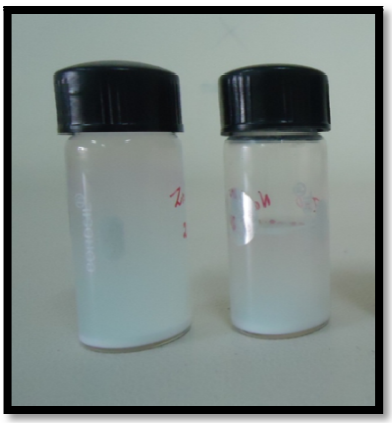


Figure 9: ZnO nanorod suspension nanorod Suspension

Figure 10: ZnO:Ag core-shell

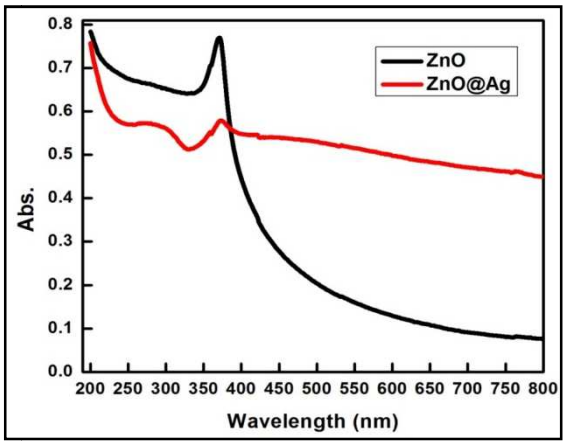


Figure 11: UV Visible spectrum of ZnO NRs and ZnO@Ag core-shell nanorods

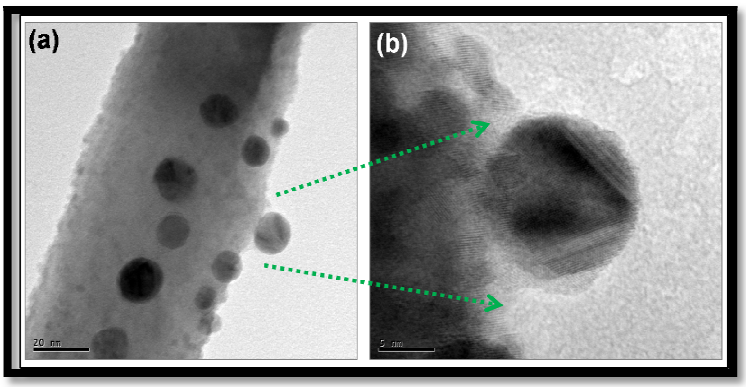


Figure 12: (a) TEM image of ZnO@Ag core-shell nanorods (b) HR-TEM image

PHASE III - CYTOTOXICITY ASSESSMENT OF SYNTHESISED EXPERIMENTAL NANOSEALERS ZnO & ZnO:Ag

The cytotoxicity of nanoparticles was evaluated by MTT assay. The reduction of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide salt (MTT) to Formosan crystal by mitochondrial dehydrogenase enzymes is the mechanism of this assay.

10×10^3 human osteoblastic cells MG63 were seeded into each well of a 96-well plate and incubated with culture medium DMEM (Dulbaccos modified eagle medium) at 37°C and 5% CO_2 overnight. Cells were treated with desired concentration of compounds in 0.01 mg, 0.05 mg & 0.1 mg for 24 hrs. 20 µL of MTT was added to each well and incubated for 3.5 h at 37°C. The media was removed carefully from each well and 150 mL of DMSO (Dimethyl sulfoxide) was added followed by agitation in an orbital shaker for 15 min. The optical density of each well was read at 590 nm using a 96 well microplate reader (Thermo Scientific™ Multiskan ™ GO Microplate Spectrophotometer). The cell viability was estimated by comparing the absorbance of the cells cultured on different scaffolds to that of the control.

Figure 13: MTT

Figure 14: 96 - well plate

Figure15:Microplate reader

PHASE IV - OBTURATION OF ROOT CANAL SAMPLES

After 21 days of incubation the roots were removed from the incubator and each root were rinsed with 5 ml of saline for 1 minute. Teeth were randomly divided into three groups – ZnO eugenol sealer, Nano ZnO sealer and Nano ZnO:Ag sealer, with seven teeth per group. Root canals were dried with paper points size 30 and 6% taper. The powder liquid component of zinc oxide eugenol sealer was mixed according to the manufacturer's instruction. With the same powder liquid ratio the Nano ZnO and Nano ZnO:Ag particles were mixed with eugenol liquid and the sealers were applied with lentulo spiral. Obturation of the teeth was done with F3 size master cone by cold lateral compaction. The excess gutta percha were seared on the coronal aspect and the orifice was sealed with cavit. All these procedures were carried out in Laminar Air Flow Chamber sterilized by UV light**(Figure 15).** The obturated teeth were stored in humidity chamber **(Figure 18)** at 100% humidity and 37°C for two weeks allowing complete setting of the sealers.

The roots were embedded in self cure acrylic resinand each root was then transversely sectioned into coronal, middle and apical third sections using hard tissue microtome (Leica, Germany)**.** Now the experimental groups contained 21 sections/samples per group.

Group A: Twenty one specimens obtained by sectioning coronal, middle and apical third roots treated with ZnO Eugenol sealer

Group B: twenty one specimens obtained by sectioning coronal, middle and apical third roots treated with Nano ZnO sealer

Group C: twenty one specimens obtained by sectioning coronal, middle and apical third roots treated with Nano ZnO:Ag sealer.

Figure 16: Laminar Airflow chamber

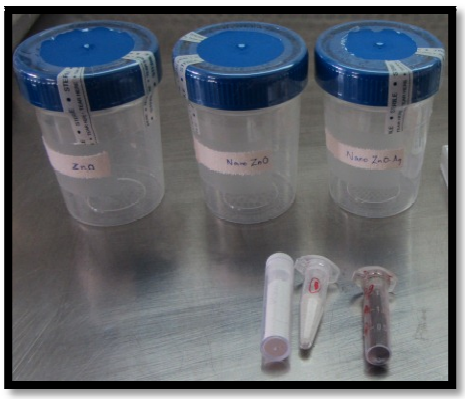


Figure 17: Synthesized experimental nano sealers

Figure 18: Obturated root samples

Figure 19: Humidity chamber

Figure 20: Elastomeric impression mold with mounted root sample in self-cureacrylic.

Figure 21: Mounted root samples

Figure 22: Hard tissue microtome samples

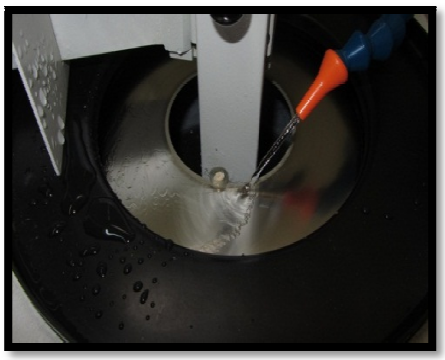


Figure 23: Sectioning of mounted root

PHASE V - CONFOCAL LASER SCANNING MICROSCOPIC VISUALIZATION OF TREATED TEETH SAMPLES

FLUORESCENT STAINING:

The fluorescent dyes Fluorescein Diacetate diluted in acetone (FDA) and Propidium Iodide (PI) (Sigma, USA) diluted in distilled water were prepared to give a concentration of 4mg/mL of Fluorescein Diacetate and 1.4mg/mL of Propidium Iodide. The root sections were washed with Phosphate buffered saline twice to remove any debris present. The root sections were placed in microcentrifugal tubes and 400 µL of FDA was added per vial in dark and the sections were kept immersed in the solution for10 minutes at room temperature. Then the roots were then removed from the vial and blotted dry and were immersed with PI for 2 minutes.

Figure 24: FDA & PI

Figure 25: Root sections after staining with fluorescent dyes

CONFOCAL LASER SCANNING MICROSCOPY:

All the sections were dried and were observed under a confocal laser scanning microscope (Zeiss, LSM 510 META, Germany) with 20X and 63 X magnifications. The fluorescent images obtained were in terms of green and red pixels, corresponding to live and dead bacteria. AIM software was used to assess the viability of E.faecalis against the various sealers used, by quantifying the bacteria individually as live and dead.

Figure 26: Confocal Laser Scanning Microscope

STATISTICAL ANAYSIS

The statistical analysis was performed with the SPSS 19 software system (IBM SPSS Statistics,Chicago, USA). Descriptive statistics was performed using One Way Anova followed by Tukey HSD (Post Hoc) with levels of significance set at $P < 0.05$.

RESULTS

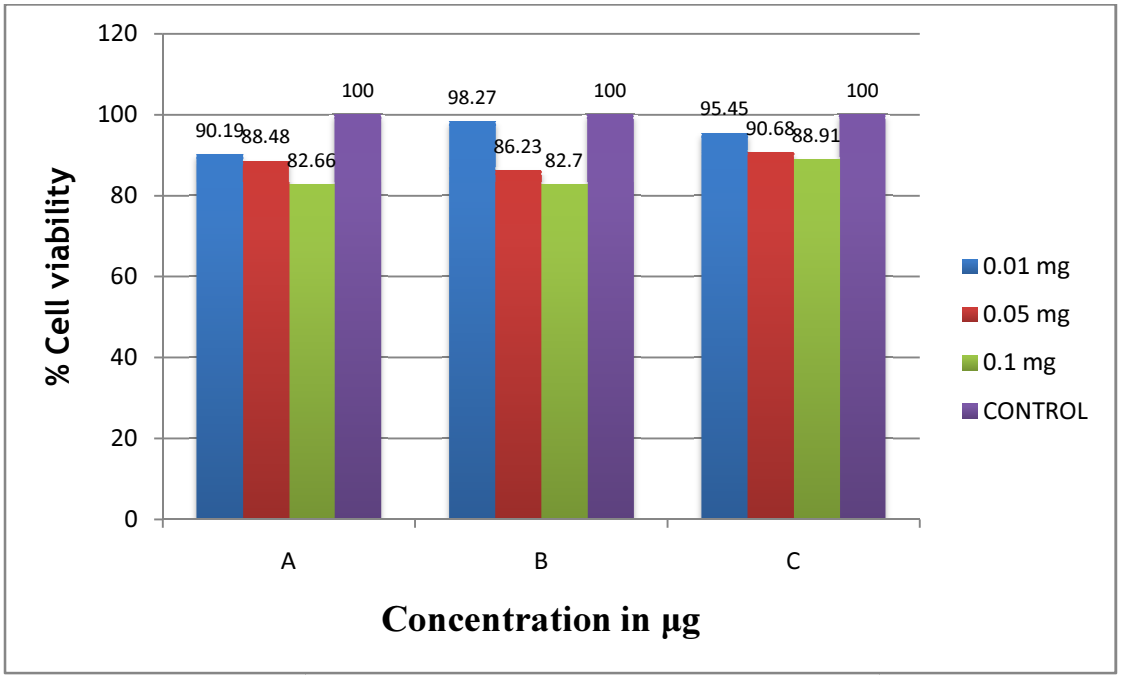
CYTOTOXIC EVALUATION OF EXPERIMENTAL NANOSEALERS ON HUMAN OSTEOBLAST LIKE MG63 CELLS

TABLE 1: RELATIVE VIABILITY OF DIFFERENT GROUPS COMPARED WITH NEGATIVE CONTROL

Group	Concentration	Mean count	% of cell viability
Control		0.3076	100.00
A	0.01mg	0.2774	90.19
	0.05mg	0.2721	88.48
	0.1mg	0.2542	82.66
B	0.01mg	0.3022	98.27
	0.05mg	0.2652	86.23
	0.1mg	0.2544	82.70
C	0.01mg	0.2936	95.45
	0.05mg	0.2789	90.68
	0.1mg	0.2735	88.91

TABLE I shows mean cell count and percentage cell viability of MG63 cell lines after treating with ZnO sealer, Nano ZnO sealer and Nano ZnO:Ag sealer at concentrations of 0.01mg, 0.05mg and 0.1mg compared with negative control (untreated cells).

GRAPH I: BAR DIAGRAM SHOWING CELL VIABILITY OF DIFFERENT GROUPS COMPARED WITH NEGATIVE CONTROL

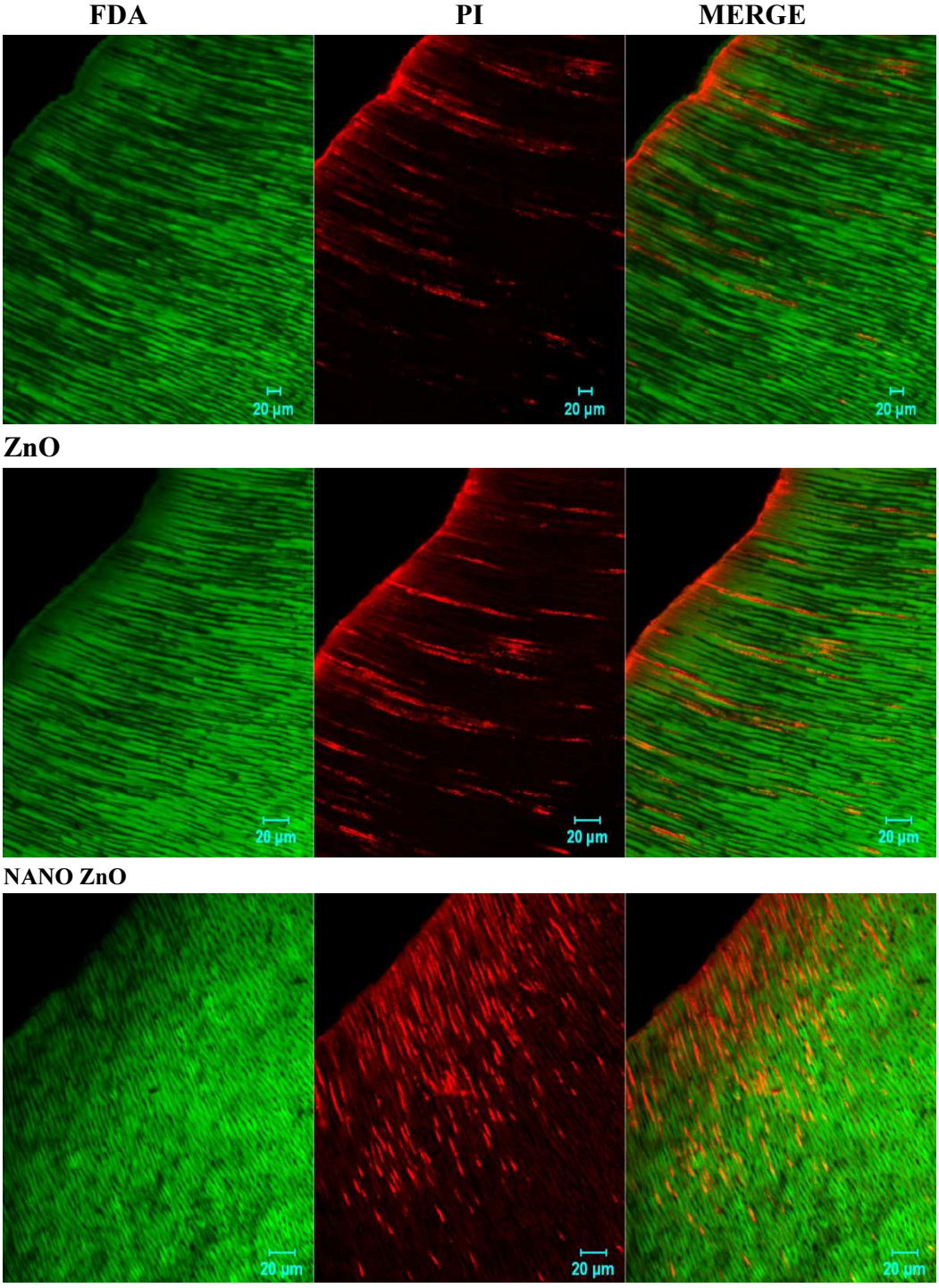


GRAPH Ishows the effect of undiluted extracts of zinc oxide, Nano zinc oxide and Nano zinc oxide silver sealers at three different concentrations of 0.01mg, 0.05mg and 0.1mg on cell viability of MG63 cells, evaluated by MTT test. Data are expressed as percentage of optical density compared with negative control (100% viability). Compared to the positive control group zinc oxide, the experimental nano sealers nano zinc oxide and nano zinc oxide silver group showed better cell viability and hence lesser cytotoxicity / better biocompatibility.

ANTIMICROBIAL EFFICACY OF EXPERIMENTAL NANOSEALERS AGAINST ENTEROCOCCUS FAECALIS – A CONFOCAL LASER SCANNING MICROSCOPIC ANALYSIS

CONFOCAL LASER SCANNING MICROSCOPY IMAGES

FIGURE 27: Bacterial viability in the coronal third segments of teeth samples (20x)

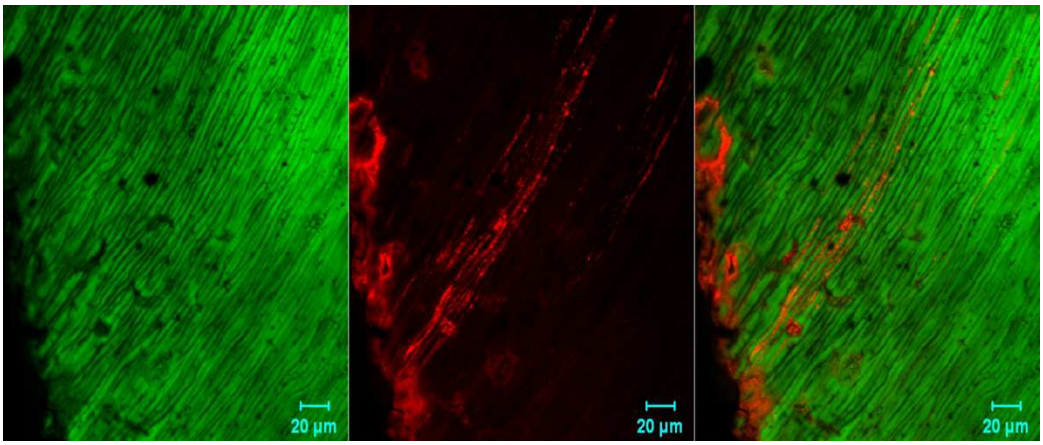


GREEN FLUORESCENCE INDICATE LIVE BACTERIA

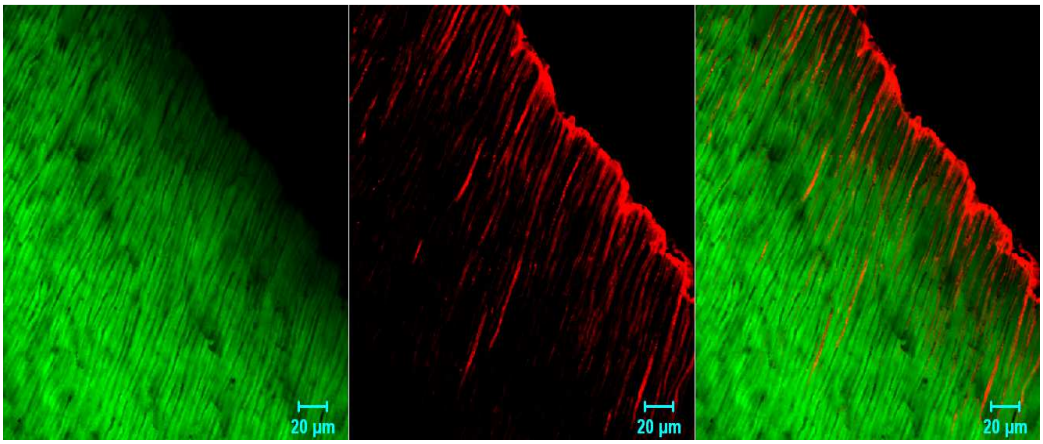
RED FLUORESCENCE INDICATE DEAD BACTERIA

Figure 28: Bacterial viability in the middle third segments of teeth sample (20x)

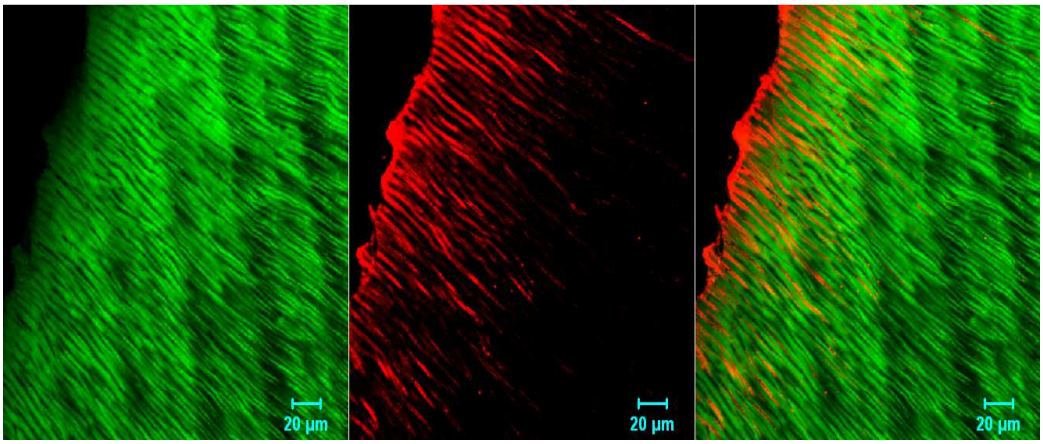
FDA **PI** **MERGE**



ZnO



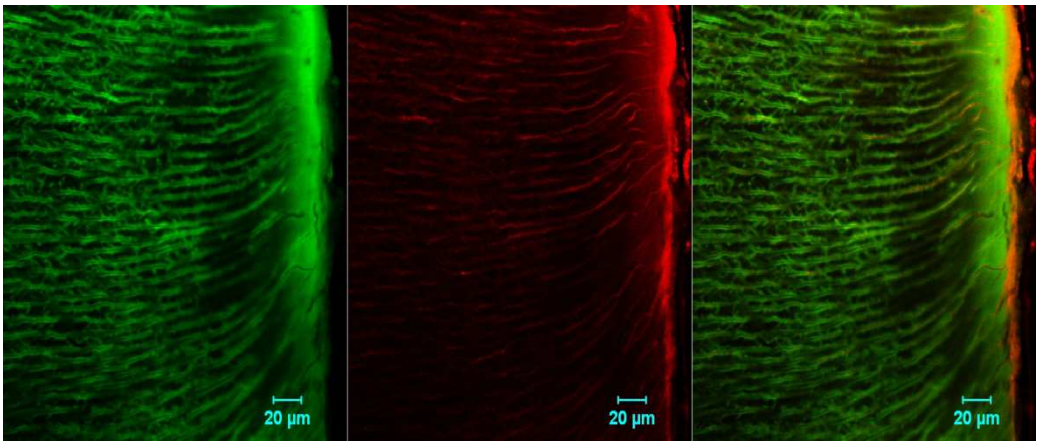
NANO ZnO



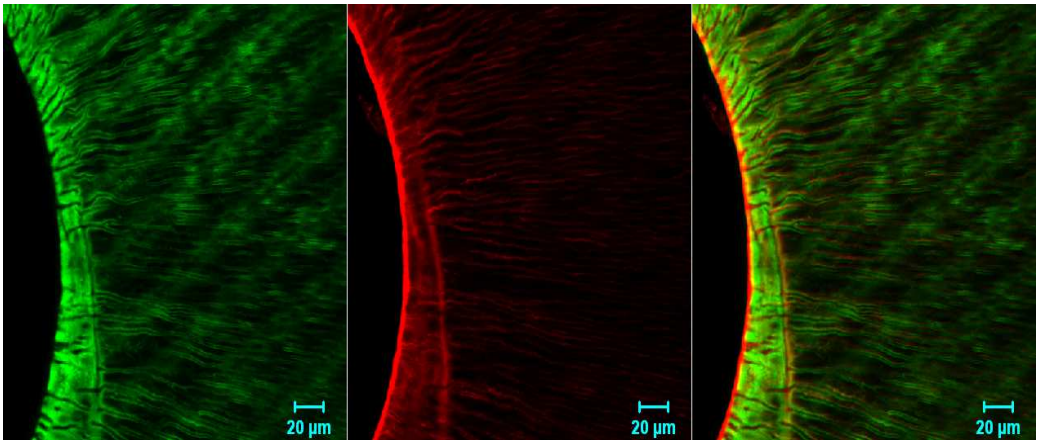
NANO ZnO:Ag

FIGURE 29: Bacterial viability in the apical third segments of teeth sample (20x)

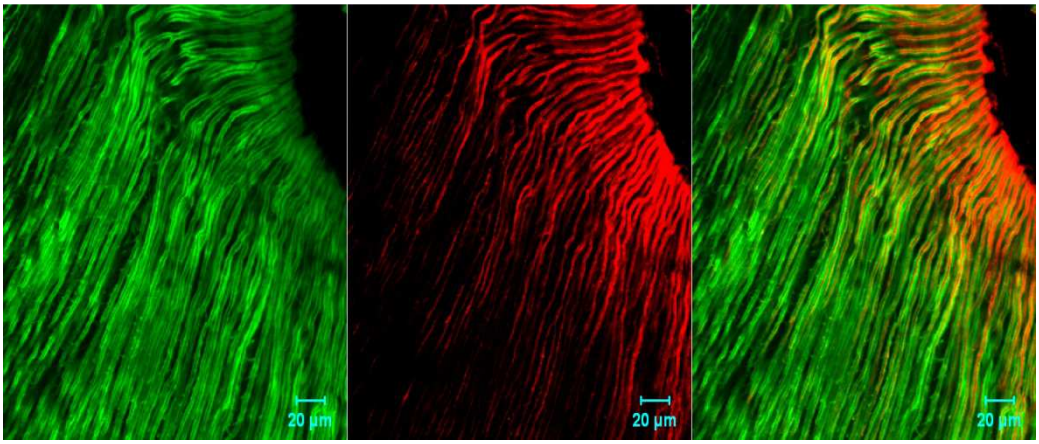
FDA **PI** **MERGE**



ZnO



NANO ZnO



NANOZnO:Ag

MASTER CHART

TABLE II: GREEN AND RED FLUORESCENCE IN PIXELS

Group	ROOT SAMPLES	CORONAL		MIDDLE		APICAL	
		GREEN	RED	GREEN	RED	GREEN	RED
A	1	22364	6625	18215	9976	10111	8877
	2	22483	9087	13735	14399	11737	6817
	3	28307	6864	14550	12655	8849	10115
	4	23412	12559	19244	19053	7577	3160
	5	21488	1598	17901	11933	9903	20017
	6	20224	5798	16357	5305	6779	3340
	7	18360	10622	19525	2619	6817	6330
B	8	18810	34041	16161	8504	11649	16063
	9	19337	18642	11329	4639	10197	7082
	10	19953	7051	14648	14749	1526	7886
	11	18961	8138	16456	14593	11839	5426
	12	19528	7821	16305	24059	1607	6853
	13	17452	19233	9909	5324	2584	3828
	14	6628	4234	9169	8743	3368	20912
C	15	10383	32974	11787	11662	14113	17507
	16	11421	22800	13346	11103	14106	10775
	17	13646	45263	10030	17678	12008	23137
	18	6076	22157	17158	16320	7457	11276
	19	7727	22566	16610	14827	3634	20135
	20	6833	32468	9167	13084	3107	12353
	21	7456	25149	9949	31854	2431	19388

Group A: ZnO

Group B: Nano ZnO

Group C: Nano ZnO@Ag

GREEN: Corresponds to live bacteria

RED : Corresponds to dead bacteria

STATISTICAL ANALYSIS

TABLE III A: ONE WAY

LIVE BACTERIA

Group	Section	Minimum	Maximum	Mean	Standard Deviation
ZnO	Coronal	18360	28307	22377.43	3104.891
	Middle	13735	19525	17075.71	2263.442
	Apical	6779	11737	8825.00	1873.717
Nano ZnO	Coronal	6628	18810	11095.00	4057.634
	Middle	9169	16456	13425.00	3197.421
	Apical	1526	11649	8482.43	4331.839
Nano ZnO:Ag	Coronal	6076	11421	9792.14	3886.608
	Middle	9167	17158	10578.29	3253.803
	Apical	2431	14113	8122.57	5242.984

TABLE III B: ANOVA

LIVE BACTERIA

Section	Group	Mean	F value	p value
Coronal	A	22377.43	16.215	**0.000 ***
	B	11095.00		
	C	9792.14		
Middle	A	17075.71	4.626	**0.024 ***
	B	13425.00		
	C	10578.29		
Apical	A	8825.00	2.075	.155
	B	8482.43		
	C	8122.57		

F value obtained by One way ANOVA test of significance
* - p value <0.05 is significant

TABLE III C: POST HOC TESTS

MULTIPLE COMPARISONS

DEPENDENT VARIABLE: LIVE BACTERIA

Section	**Group**	**Group**	**Mean difference**	**p value**
Coronal	A	B	11281.429*	**0.000 ***
		C	12585.286*	**0.029 ***
	B	C	1303.143*	**0.026 ***
Middle	A	B	3649.714	.078
		C	6497.429*	**0.027 ***
	B	C	2847.714	.853

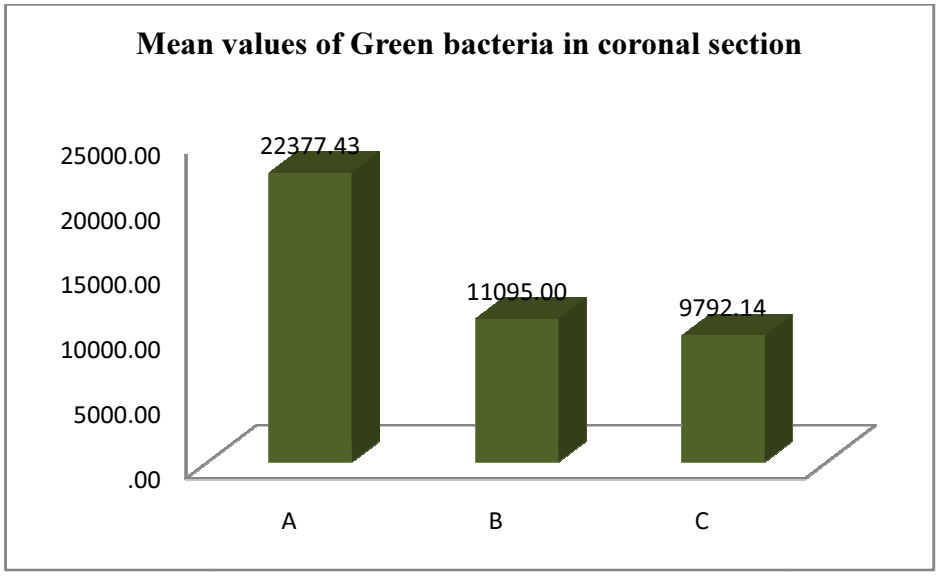
TABLE III A shows mean values of viable bacteria. Among the three groups (ZnO, Nano ZnO and Nano ZnO@Ag), the mean amounts of live bacteriaare lesser for Group C in all the three sections, indicating that Nano ZnO@Ag had less live bacteria compared to other groups.

IN **TABLE III B**, the F value 16.215 and 4.626 for the mean difference in the amount to live bacteria in the coronal and middle third sections of the three groups are significant ($p < 0.05$). No significant difference is seen in the amount to live bacteria in the apical section.

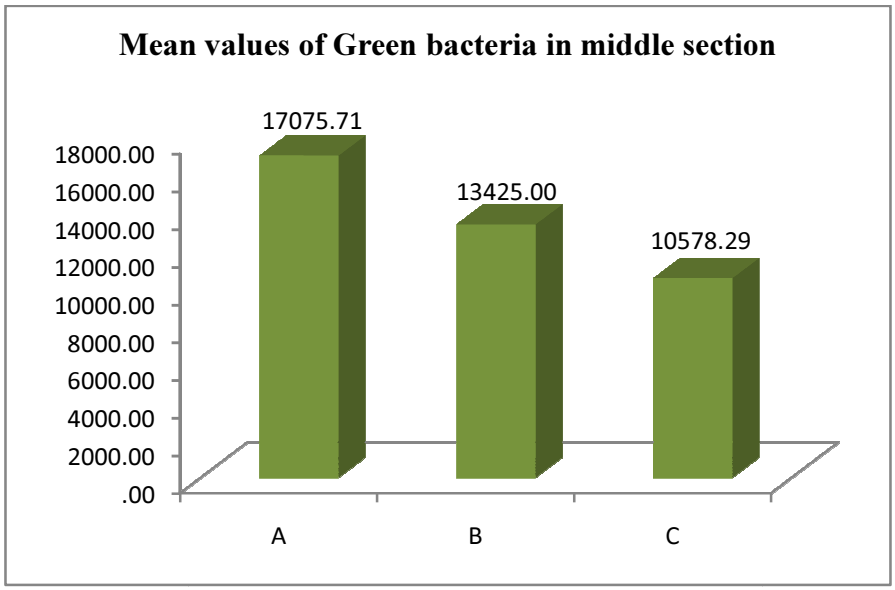
IN **TABLE III C**, the post hoc analysis reveals that Nano ZnO@Ag is more effective when compared to the other two sealers.

GRAPH II: BAR DIAGRAM REPRESENTING MEAN VALUES OF LIVE BACTERIA BETWEEN ZnO, N ZnO& N ZnO:Ag

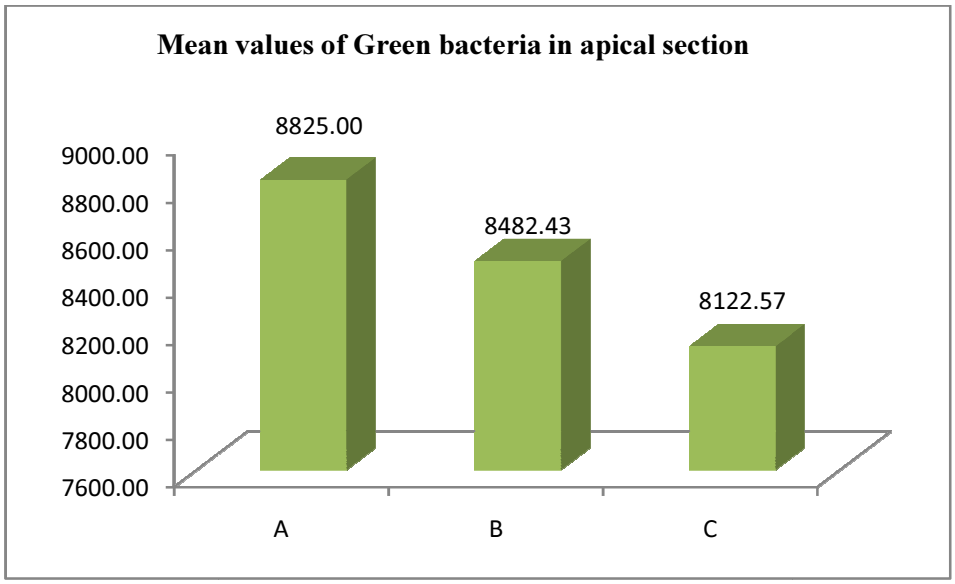
GRAPH II A



GRAPH II B



GRAPH II C



GRAPH II – The mean values of viable bacteria shows statistically significant difference between Group C with Group A and Group B in coronal third section and between Group C and Group A in middle third sections.

TABLE IV A: ONE WAY

DESCRIPTIVES

DEAD BACTERIA

Group	Section	Minimum	Maximum	Mean	Standard Deviation
ZnO	Coronal	1598	12559	7593.29	3575.345
	Middle	2619	19053	10848.57	5531.362
	Apical	3160	20017	8379.43	5744.163
Nano ZnO	Coronal	4234	34041	14165.71	10540.306
	Middle	4639	24059	11515.86	6826.941
	Apical	3828	20912	9721.43	6287.433
Nano ZnO:Ag	Coronal	22157	45263	29053.86	8502.723
	Middle	11103	31854	16646.86	7118.243
	Apical	10775	23137	16367.29	4894.856

TABLE IV B: ANOVA

DEAD BACTERIA

Section	Group	Mean	F value	p value
Coronal	A	7593.29	12.942	**0.000 ***
	B	14165.71		
	C	29053.86		
Middle	A	10848.57	1.653	.219
	B	11515.86		
	C	16646.86		
Apical	A	8379.43	3.982	**0.037 ***
	B	9721.43		
	C	16367.29		
F value obtained by One way ANOVA test of significance * - p value <0.05 is significant				

TABLE IV C: POST HOC TEST

MULTIPLE COMPARISONS

DEPENDENT VARIABLE: DEAD BACTERIA

Section	Group	Group	Mean difference	p value
Coronal	A	B	-6572.429	.305
		C	-21460.571	**0.000 ***
	B	C	-14888.143	**0.008 ***
Apical	A	B	-1342.000	.898
		C	-7987.857	**0.042 ***
	B	C	-6645.857	.100

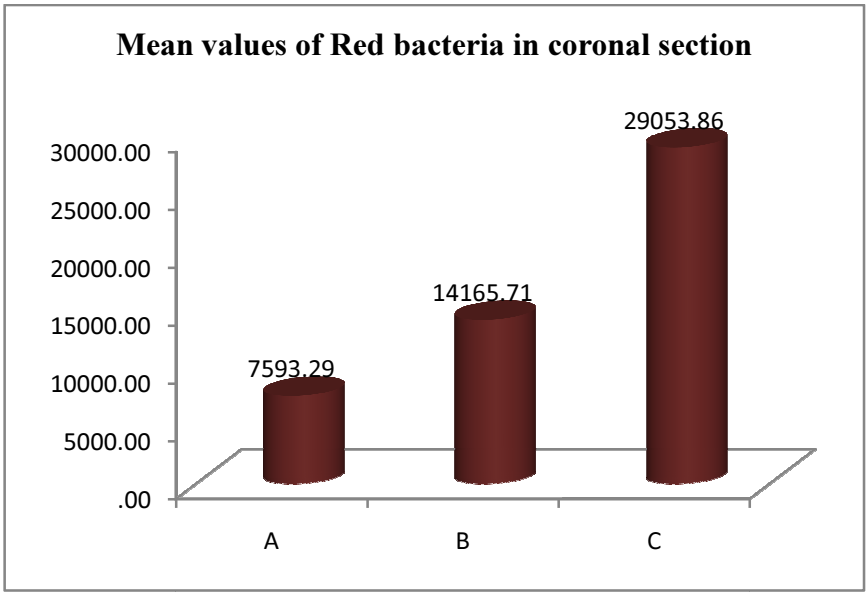
TABLE IV A shows mean values of dead bacteria. Among the three groups (ZnO, Nano ZnO and Nano ZnO@Ag), the mean amounts of dead bacteria are greater for Group C in all the three sections, indicating that Nano ZnO@Ag had more dead bacteria compared to other groups.

IN **TABLE IV B,** the F value 12.942 and 3.982 for the mean difference in the amount to dead bacteria in the coronal and apical third of the three groups is significant ($p < 0.05$). No significant difference is seen in the amount to dead bacteria in the middle third section.

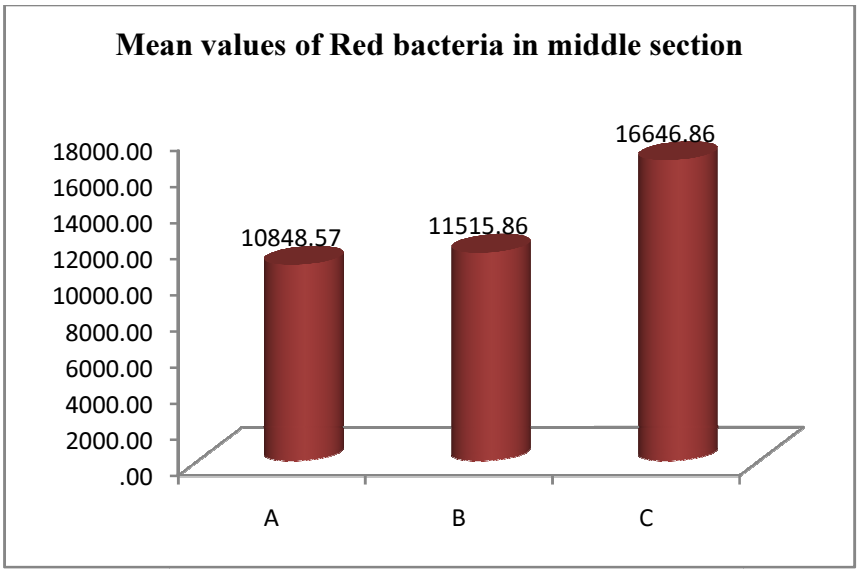
IN **TABLE IV C**, the post hoc analysis reveals that Nano ZnO@Ag is more effective when compared to the other two sealers.

GRAPH III: BAR DIAGRAM REPRESENTING MEAN VALUES OF DEAD BACTERIA BETWEEN ZINC OXIDE, NANO ZINC OXIDE AND NANO ZINC OXIDE SILVER

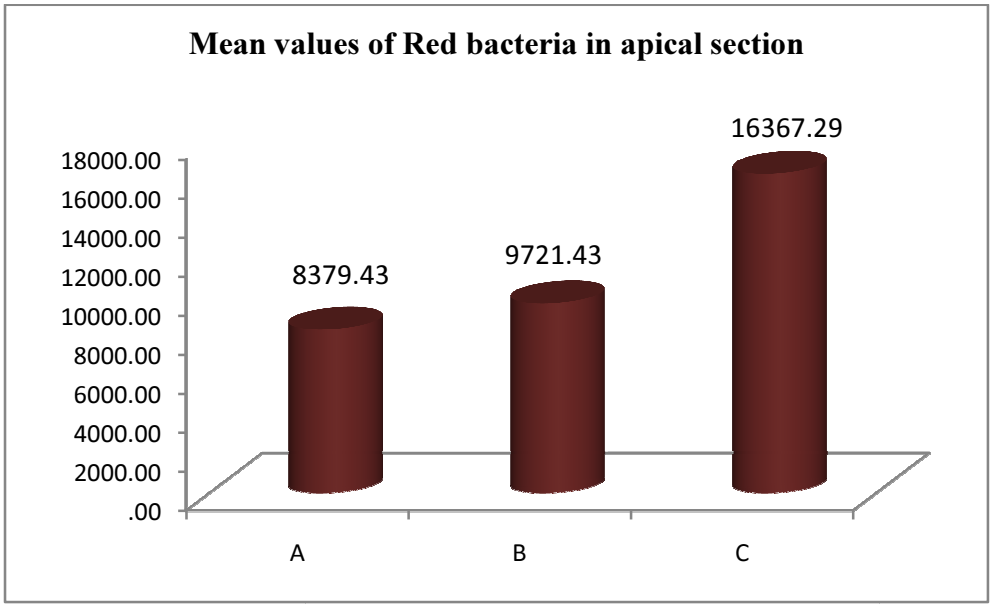
GRAPH III A



GRAPH III B



GRAPH III C



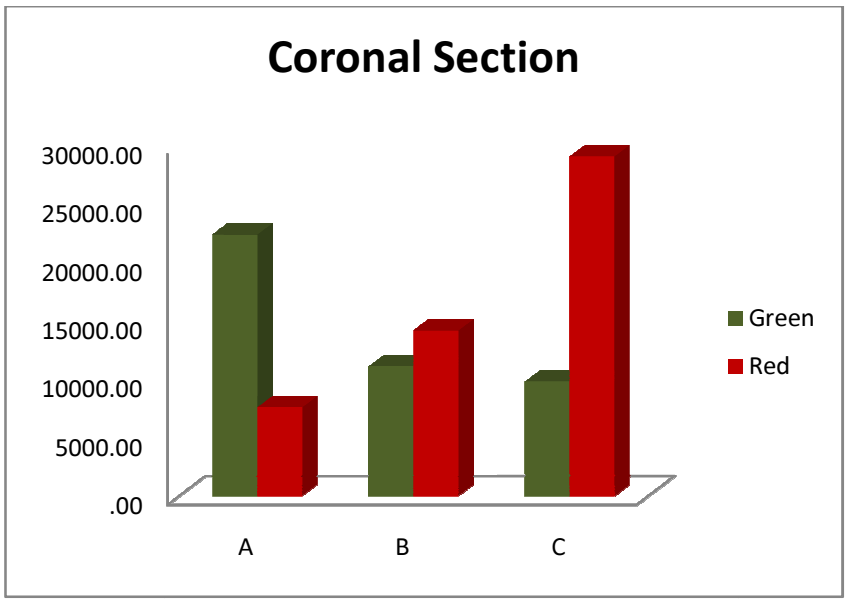
GRAPH III – The mean values of dead bacteria shows statistically significant difference between Group C with Group A and Group B in coronal third sections and between Group C and Group A in apical third sections.

TABLE V: RATIO BETWEEN LIVE AND DEAD BACTERIA IN CORONAL, MIDDLE AND APICAL THIRD SECTIONS OF THREE GROUPS

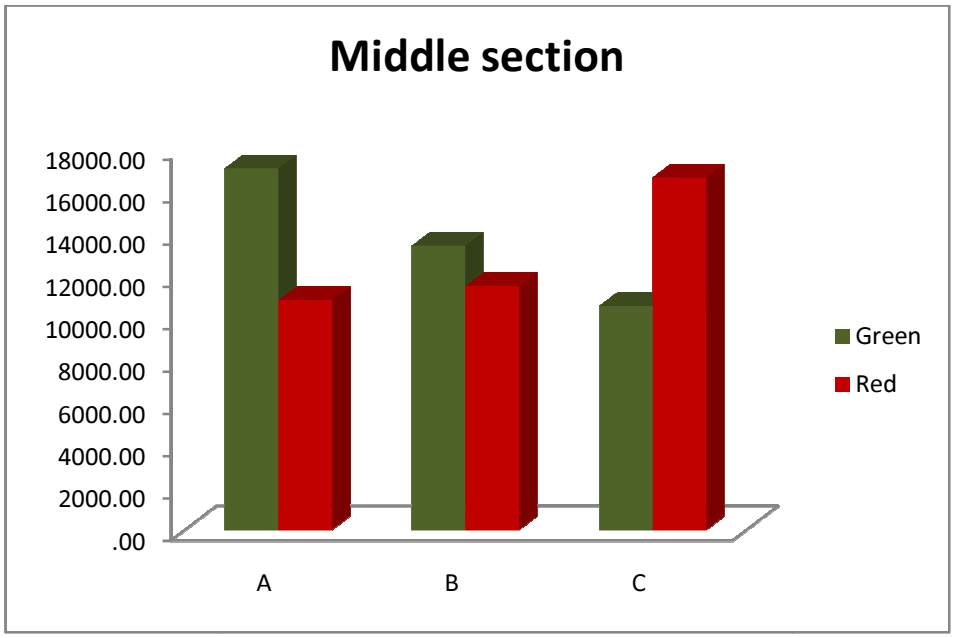
	CORONAL	MIDDLE	APICAL
A	22377.43/7593.29	17075.71/10848.57	8825.00/8379.43
B	11095.00/14165.71	13425.00/11515.86	8482.43/9721.43
C	9792.14/29053.86	10578.29/16646.86	8122.57/16367.29

GRAPH IV: GRAPHICAL REPRESENTATION OF LIVE AND DEAD BACTERIA PRESENT IN THREE GROUPS

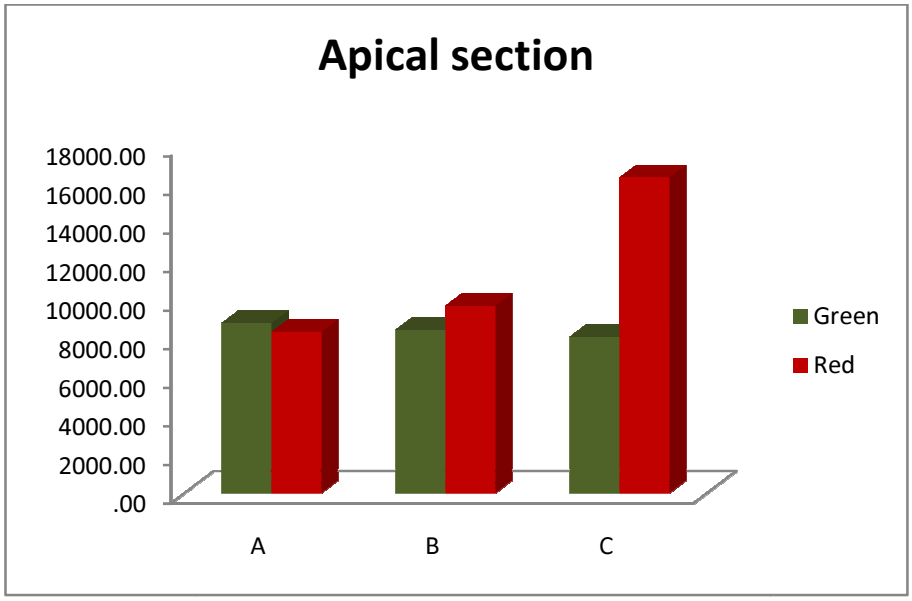
GRAPH IV A



GRAPH IV B



GRAPH IV C



GRAPH IV shows lesser viable bacteria and more dead bacteria in Group C in all the three sections.

DISCUSSION

Chemomechanical preparation is of utmost importance in successful endodontic treatment. However, this does not negate the importance of the quality of obturation in which the sealer has a role to play. According to Grossman, An ideal endodontic sealer should be biocompatible and dimensionally stable; it should seal well and have a strong, long-lasting antimicrobial effect[52]. Unfortunatly, it is difficult to produce sealers with proper physicochemical properties and biological compatibility. Materials that are well tolerated by tissues compromise sealer properties, and vice versa[53].

Clinical evidence shows that mechanical instrumentation leaves significant portions of root canal wall untouched[54]. Apart from being a nidus for bacterial re-infection, this also can serve as nutritional source for the remaining microorganisms. Hence, a three dimensional seal with the antimicrobial property of the sealer is critical for endodontic success. A sealer with an antimicrobial activity can be considered advantageous, in order to eliminate the remaining microbes present in the root canal after chemo-mechanical preparation of the root canal system and to prevent reinfection.

Zinc oxide eugenol based sealers have been traditionally the most commonly employed sealers during root canal treatment because it reasonably meets most of the Grossmann's requirements for sealers. The antimicrobial effect of zinc oxide eugenol cement was mainly attributed to the action of eugenol. Eugenol, a phenolic compound acts on microorganisms by protein denaturation whereby the protein becomes non-functional[55,56]. The results of the various studies performed by **Markowitz K et al. (1992)**[57] and **Saggar V et al.(1996)**[58]also confirms that zinc oxide eugenol containing sealers were superior in inhibiting the microorganisms. However, the drawbacks with the traditional zinc oxide eugenol sealer are its solubility and tissue reactions when it comes in contact with periapical tissues.

In recent years, various nanoparticles have gained popularity as antimicrobial agents due to their broad spectrum of activity and biocompatibility[59]. Recent studies have focused on using nanoparticulate materials as root canal sealers[26,60]. Nanoparticles have attracted attention in endodontics, because of their better penetration into the dental tubules, profound antibacterial properties and decreased microleakage[60]. Nanoparticulates exhibit superior antibacterial activity as a result of their polycationic/polyanionic nature with higher surface area and charge density, resulting in greater degree of interaction with the bacterial cell[61] and also the size of nanoparticulates plays an important role in their antibacterial activity, with smaller particles showing higher antibacterial activity than the macroscaled ones[62-64].

With this view, we indigenously synthesized two experimental nanosealers – Nano ZnO & Nano ZnO:Ag sealers at PSG institute of advanced studies, Nano sensors laboratory, Coimbatore, India and tested their biocompatibility with human osteoblast like MG63 cells and antimicrobial efficacy against E.faecalis.

For many years, zinc oxide containing sealers have been the most popular and widely used sealers. Various modifications have been made to overcome its drawbacks such as slow setting time, shrinkage on setting, solubility[65] and staining the tooth structure[66] and now there are many formulations that are available commercially that have zinc oxide as the primary ingredient. In this study the modification we made to improvise zinc oxide based sealers was by synthesizing Nano ZnO particles.

Antibacterial activity of polycationic ZnO nanoparticle could be attributed to the electrostatic attraction with the negatively charged bacterial cell, which might lead to the altered cell wall permeability, resulting in leakage of the proteinaceous and other intracellular components and death of the cell[67]. Metallic nanoparticulates (ZnO) are

also known to cause membrane damage as a result of lipid peroxidation by the reactive oxygen species (ROS) such as superoxide ($O2^{-}$) and hydroxyl radicals (OH^{-})[68]. Direct or close contact between the nanoparticulates and the bacterial membrane appeared to be essential for the ROS toxicity to be effective in peroxidation[69]. **Javidi et al 2014**[26] studied zinc oxide nano-particles as sealer in endodontics and its sealing ability and reported that the synthesized zinc oxide nano-powders are suitable for use as a sealer in root canal therapy to prevent leakage. The antibacterial potential of Nano ZnO has been studied by certain authors against E.faecalis. **Shrestha et al 2010**[24] found that chitosan nanoparticle and zinc oxide nanoparticle possess a potential antibiofilm capability against E.faecalis biofilm. **Guerreiro-Tanomaru et al 2013**[25] showed nanoparticulate ZnO possessed better antimicrobial effect against E.faecalis than microparticulate ZnO but lesser efficacy than combination of Chlorhexidine with micro ZnO particle.

The other test material was Nano ZnO particles doped with Nano Ag clusters. In earlier days, among the various modifications made with conventional zinc oxide based sealers to improve its characteristics, Rickert's formula was one such modified formulation. Rickert's formula as powder/liquid sealer contained silver particles for radiopacity. Although it was possible to demonstrate the presence of lateral and accessory canals, the sealer had the distinct disadvantage of staining the tooth structure. With the advent of nanotechnology into dentistry the materials are being reduced to nanoscale with improved and unique properties which is very different from their micro counterparts. Likewise converting the Ag particles from micro to nano scale would result in improved properties and help to overcome its staining characteristics. **Makkar s et al2014**[28] evaluated the antimicrobial effect of silver Nano particle based irrigants as endodontic irrigant in comparison with NaOCl against E.faecalis and found silver nano

particle based irrigant to be more efficacious than NaOCl. In another study **Lotfi et al 2011**[27] compared the antibacterial efficacy of nanosilver (NS), chlorhexidine gluconate (CHX) and sodium hypochlorite (NaOCl) against Enterococcus faecalis and found that NS in a remarkably lower concentration would possess the same bactericidal effect as 5.25% NaOCl.

The biologic effects of silver are believed to be closely related to silver ion[70,71]. In an aqueous microenvironment, silver nano-particles (AgNPs) continuously release silver ion[72]. It is well known that smaller silver nano-particles show stronger and better bactericidal effect than larger particles because they have a larger surface area for interaction[73]. Binding to essential cellular structural elements like enzymes and other proteins[74], particularly to their SH-groups and interfering with the integrity of the bacterial cell[75] are the main reasons for bactericidal properties of silver ions. The AgNPs interact with multiple targets in the microbial cell, such as cell membrane, enzymes, and plasmids, simultaneously providing the bacteria least capacity to gain resistance.

To further enhance the antibacterial properties of the ZnO Nanoparticles, we doped the nanoparticles with silver nanoclusters[76]. When the silver nanoparticles have been added to the endodontic sealer formulations they showed improved antimicrobial actions and also it did not deteriorate the flow characteristics of the sealer[77].

When a new dental material is synthesized, its biocompatibility should be determined. Any nano endodontic sealer must remain compatible with periapical tissues during long-time contact[78]. The direct contact of root canal filling materials with periapical tissues could result in a bone tissue reaction, mediated partially by osteoblasts[79,80]. Thus it is important to evaluate the biocompatibility of root filling

material on osteoblast-like cells such as MG63[81]. **Xu et al. 2010[82]** in their study compared the cytotoxicity of RealSeal root canal filling material and epoxy resin-based root canal sealer (AH Plus) and gutta-percha by using human osteoblast-like MG63 cells and concluded that the RealSeal sealer was significantly more toxic to MG63 cells than the AH Plus within 3 days, whereas RealSeal core material was similar with gutta-percha. In another study **Attik et al 2014[83]** compared the in vitro biocompatibility of Biodentine and White ProRoot mineral trioxide aggregate (MTA) with MG63 osteoblast-like cells and concluded that biocompatibility of Biodentine to osteoblast like cells was comparable to MTA.

In this study, Synthesized Nano Zinc oxide and Nano Zinc oxide doped with Nano silver clusters were checked for its biocompatibility using MTT assay on human osteoblast like MG63cells. According to the methods reported in the ISO 7405 and the ISO 10993-5 standards, there are basically two approaches to the in vitro evaluation of the cytotoxicity of experimental cements. Either the material directly placed in contact with cells, or the liquid extract of material placed in contact with cells. In our study we tested the material by placing it in direct contact with the cells. As recommended by the ISO (10993-5, 2009), if relative cell viability is less than 70%, the test materials would be considered as cytotoxic to that particular cell type[84]. Survival of the target cells after direct culturing in different concentration of test materials was estimated with MTT assay. Nano ZnO particles showed cell viability of 98.27% at concentration of 0.01mg, 86.23% at concentration of 0.05 mg and 82.70% at concentration of 0.1 mg. Nano ZnO@Ag particles showed cell viability of 95.45% at concentration of 0.01 mg, 90.68% at concentration of 0.05 mg and 88.91% at concentration of 0.1mg (TABLE I). Both the test materials showed biocompatibility better than the positive control material

– the micro Zinc oxide particle sealer which showed cell viability of 90.19% at concentration of 0.01 mg, 88.48% at concentration of 0.05mg and 82.66% at concentration of 0.1mg.

Enterococcus faecalis has been found to be one of the most persistent microorganisms, with a prevalence of 24 to 77% in root filled teeth with periradicular lesions[85]. It has a potential to adhere to both collagen and hydroxyapatite of dentin in the presence of human serum and also possesses virulence factors such as lytic enzymes, cytolysin, aggregation substance, pheromones and lipoteichoic acid, which facilitates its survival in the root canal[49]. Studies have shown E.faecalis to be viable inside the root canal dentin up to a period of 12 months[86]. Therefore, it is important to consider treatment regimens aimed at eliminating or preventing the infection of E.faecalis during all the phases of root canal treatment and hence E.faecalis was the microorganism of choice for the current study.

Bacterial viability assessment was done using a confocal laser scanning microscope which has advantages of visualization, differentiation of live and dead microorganisms and quantification individually, over the conventional methods. Confocal microscopy has been previously used in endodontics to investigate the adaptation and percentage of penetration of endodontic sealers into root dentin[87,88], which has been shown to be a simple method using Rhodamine dye in a non-dehydrated samples. Now CLSM is used in several microbiological studies and 3D reconstructions can also be generated with the digital data[89]. **Zapata et al (2008)[50]** in their study explored the potential of confocal laser scanning microscopy for in situ identification of live and dead Enterococcus faecalis in infected dentin and found it to be useful in discriminating viable and dead bacteria in infected dentin after staining

with FDA/PI. **Parmar et al (2011)[51]** assessed the capacity of confocal laser scanning microscopy to differentially image and quantify viable and non-viable bacteria within mineralized tissueand showed it to be a convenient and reproducible approach for assessing viability of the bacteria and the extent of bacterial penetration into the dentinal tubules.

CLSM necessitates the use of fluorescent binding agents to make visualization possible by the property of fluorescence. Such agents used in the current study are fluorescein diacetate (FDA; Sigma, St Louis, MO) and propidium iodide (PI, Sigma). FDA is a nonfluorescent cell permeable dye that is converted to fluorescein (green) by intracellular esterases produced by metabolically active microorganisms[90-92]. PI is a fluorescent molecule impermeable to the cellular membrane and generally excluded from viable cells; thus, live bacterial cells are fluorescent green, whereas dead bacteria with damaged membranes are fluorescent red[93]. The dentin segments were examined on an inverted Leica TCS-SPE confocal microscope (Leica Microsystems GmbH, Mannheim, Germany). The respective absorption and emission wavelengths were 494/518 nm for fluorescein diacetate and 536/617 nm for PI. The CLSM analysis used in this study has advantages over the conventional fluorescence microscopy to visualize bacteria in dentinal tubules such as better image resolution and the ability to eliminate scattered or out-of-focus light and to show individual bacterial cells inside dentinal tubules.

After the confocal laser scanning microscopic analysis, the values obtained for each sealer used was tabulated. In our study, (TABLE V) the results indicated that Nano ZnO:Ag sealer (with a mean value of Live/Dead: Coronal 3rd-9792.14/29053.86, Middle 3rd – 10578.29/16646.86, Apical 3rd – 8122.57/16367.29) was more efficacious

against E.faecalis followed by Nano Zno sealer (with a mean value of Live/Dead : Coronal 3rd – 11095.00/14165.71, Middle 3rd – 13425.00/11515.86, Apical 3rd – 8482.43/9721.43) and ZnO sealer (with a mean value of Live/Dead : Coronal 3rd – 22377.43/7593.29, Middle 3rd – 17075.71/10848.57, Apical 3rd – 8825.00/8379.43) at a level of significance of $p< 0.05$. Significant difference in reduction of live bacteria was found between the three groups in coronal and middle third sections, whereas significant difference in dead bacterial count was found between the three groups in coronal and apical third sections. Also complete elimination of E.faecalis was not achieved with all the three root canal sealers used.

In the current study, Nano ZnO:Ag sealer showed higher efficacy against E.faecalis compared to Nano ZnO and ZnO sealer. This may be due to the synergistic effect of Nano zinc oxide doped with Nano silver clusters. Another factor that could have influenced the results is the penetration ability of the Nano sealers deep into the dentinal tubules compared to the traditional zinc oxide sealer due to their Nano particle size.

Within the limitations of the current study, it can be concluded that the tested experimental nanosealers are biocompatible with Nano ZnO:Ag sealer to be more effective than Nano ZnO sealer and conventional ZnO sealer in the elimination of E.faecalis from the root canal system. And also, fluorescent viability staining is a convenient and accurate method for quantification of live and dead bacteria in human dentinal tubules. Future research with regard to mixing proportions and methodology relating to in vivo conditions can help in understanding and improving the efficacy of these experimental nanosealers.

SUMMARY

The study involved three phases,

PHASE I : **Synthesis** of experimental nanosealers Nano ZnO & Nano ZnO@Ag and,

Characterization of the synthesized sealers using Tranmission Electron Microscopy (TEM) and UV Spectroscopy

PHASE II : **Cytotoxicity analysis** of the synthesized sealers using MTT assay on Human Osteoblast like MG63 cell lines

PHASE III : **Antimicrobial efficacy** of the synthesized sealers analyzed against Enterococcus faecalis using Confocal Laser Scanning Microscopy

ZnO Nanorods were synthesized by hydrothermal method using the raw chemicals Zinc chloride, Potassium hydroxide, Sodium borohydride, Tri-sodium citrate, Silver nitrate and 3-Aminopropyl-trimethoxysilane. Synthesized ZnO nanorods were surface functionalized using 3-aminopropyl-trimethoxysilane (APTMS) bifunctional ligand as a preparatory phase for synthesizing ZnO@Ag Hybrid core-shell nanorods following which ZnO@Ag nanoclusters were synthesized by seed mediated growth method using Tri-ethyl amine, reducing agent.

Thus synthesized ZnO nanorods and ZnO@Ag hybrid core-shell nanorods were surface characterized using UV spectroscopy and Tranmission Electron microscopical methods to confirm their nanostructures.

After synthesizing these experimental nanosealers they were subjected to cytotoxicity assessment using MTT assay on Human Osteoblast like MG63 like cell lines in a 96 well plate. The optical density of each well after treating the human

osteoblast like cells with desired concentration of the test material (0.01mg, 0.05mg and 0.1mg) and the reagent MTT was read at 590 nm using a 96 well microplate reader (Thermo Scientific™ Multiskan ™ GO Microplate Spectrophotometer). The cell viability was estimated by comparing the absorbance of the cells cultured on different scaffolds to that of the control.

Twenty one single-rooted human mandibular premolars were decoronated, debrided and following cleaning and shaping, were infected with Enterococcus faecalis, with weekly nutrient replenishment for three weeks and then were randomly grouped into three groups consisting of 7 teeth each. The teeth were then obturated with F3 master cone by cold lateral compaction and stored in humidity chamber at 37º c and 100% humidity for two weeks allowing the sealer to set completely.

Group I – Conventional ZnO sealer

Group II – Nano ZnO sealer

Group III – Nano ZnO@Ag sealer

Following incubation, the rootswere sectioned transversely at the coronal, middle and apical regions using a hard tissue microtome. The sections were washed thoroughly and stained with fluorescent DNA binding reagents (fluorescent diacetate and propidium iodide). The bacterial viability was examined by Confocal laser scanning microscopy. Computer assisted determinations of fluorescence for live and dead bacteria were analyzed and were compared statistically.

The results of the study on biocompatibility showed that the ZnO nanorods and ZnO@Ag Hybrid core-shell nanorods were tolerated better than the control, ZnO microparticles. The results on antimicrobial efficacy showed that the complete

elimination of E.faecalis was not achieved with all the three sealers used. Nano ZnO@Ag sealer was shown to be most efficacious against E.faecalis compared to Nano ZnO sealer and conventional ZnO sealer.

CONCLUSION

Within the limitations of the current study, it can be concluded that

1. Experimentally synthesized ZnO and ZnO@Ag nanoparticles showed characteristic nano rod and hybrid core-shell nanorod appearance respectively.
2. All the experimental nanosealers were biocompatible.
3. Nano ZnO@Ag sealer was more effective than Nano ZnO sealer and Conventional ZnO sealer in the elimination of E.faecalis from the root canal system.

However, further studies should be carried out to customize synthesis and characterization of Nano sealers for endodontic applications in vivo.

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